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Mammalian Myosin Iα, Iβ, and Iγ: New Widely Expressed Genes of the Myosin I Family

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Abstract. A polymerase chain reaction strategy was devised to identify new members of the mammalian myosin I family of actin-based motors. Using cellular RNA from mouse granular neurons and PC12 cells, we have cloned and sequenced three 1.2-kb polymerase chain reaction products that correspond to novel mammalian myosin I genes designated MMIα, MMIβ, MMIγ. The pattern of expression for each of the myosin I's is unique: messages are detected in diverse tissues including the brain, lung, kidney, liver, intestine, and adrenal gland. Overlapping clones representing full-length cDNAs for MMIα were obtained from mouse brain. These encode a 1,079 amino acid protein containing a myosin head, a domain with five calmodulin binding sites, and a positively charged COOH-terminal tail. In situ hybridization reveals that MMIα is highly expressed in virtually all neurons (but not glia) in the postnatal and adult mouse brain and in neuroblasts of the cerebellar external granular layer. Expression varies in different brain regions and undergoes developmental regulation. Myosin I's are present in diverse organisms from protozoa to vertebrates. This and the expression of three novel members of this family in brain and other mammalian tissues suggests that they may participate in critical and fundamental cellular processes.

THE myosin I family of proteins, like conventional muscle myosins (myosin II), are actin-activated ATPases that generate mechanochemical force within the cell (Korn and Hammer, 1989; Pollard et al., 1991). Unlike the bipolar filaments of myosin II, myosin I's in solution exist as monomeric globular proteins. All myosin I's identified share two important structural properties: a myosin head that contains sites for ATP and actin binding, and a COOH-terminal domain that binds to membranes through interaction with negatively charged phospholipids (Adams and Pollard, 1989). Since the initial report of myosin I in Acanthamoeba (Pollard and Korn, 1973), the number and diversity of myosin I family members have grown. Although myosin I's have been well studied in organisms including Acanthamoeba (Jung et al., 1987; Lynch et al., 1989), Dictyostelium (Jung et al., 1989; Jung and Hammer, 1990; Titus et al., 1989), and Drosophila (Montell and Rubin, 1989; Hicks and Williams, 1992), only a single myosin I gene in vertebrates has been cloned and extensively characterized to date (Hoshimaru and Nakanishi, 1987; Garcia et al., 1989). This species appears to be localized exclusively to the brush border of the small intestine (Hoshimaru et al., 1989; Bikle et al., 1991) and has been designated brush border myosin I (BBMI).[1] Recent biochemical findings have pointed to the presence of additional mammalian myosin I family members (Coluccio, 1991; Barylko et al., 1992).

Biochemical, immunolocalization, and EM studies have suggested that myosin I's play diverse and important roles within cells (for review see Korn and Hammer, 1990; Hammer, 1991; Pollard et al., 1991; Cheney and Mooseker, 1992). For example, in Dictyostelium, myosin I was localized to the leading edge of motile cells and to the phagocytic cup upon ingestion of bacteria (Fukui et al., 1989). Similarly, in Acanthamoeba, myosin IC was found both at the plasma membrane and the contractile vacuole (Baines and Korn, 1990), and in chick intestinal microvilli, BBMI forms the crossbridge between the actin bundle core and the plasma membrane (Mooseker and Tilney, 1975). In addition, functional loss of the single myosin II gene in Dictyostelium did not cause defects in pseudopod extension, cell motility, phagocytosis, or retrograde movement of membrane antigens (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Jay and Elson, 1992), all actin-based activities involving interaction with the plasma membrane that could be directed by myosin I's.

We have approached our studies of myosin I's with an interest in their potential roles within the vertebrate nervous system. Cell migration, neurite outgrowth, and vesicular transport are all processes that might involve myosin I's (Mitchison and Kirschner, 1988; Smith, 1988; Pollard et al., 1991) and that are central to neuronal development. Myosin I-like proteins have been localized to growth cones by immunostaining of a neuronal cell line (Miller et al., 1992) and by immunoblotting of fractions enriched in growth cone membranes (Phelan et al., 1991). To begin to address the is-

1. *Abbreviations used in this paper*: BBMI, brush border myosin I; EGL, external granular layer.

sue of the role of myosin I's in the brain, we have devised and implemented a PCR-based strategy to clone and characterize novel myosin I genes expressed in the nervous system.

Portions of this work have been presented previously in abstract form (Sherr and Greene. 1991. *J. Cell Biol.* 115:331*a*; and Joyce et al. 1992. *Soc. Neurosci. Abstr.* 18:221).

Materials and Methods

Generation of PCR Products

The degenerate primers used in this study and their corresponding conserved amino acid motifs were as follows: 5'-ATATCTAGAAGCTTG-GWGCIGGIAARACNGARGC-3' (GAGKTEA); 5'-ATATCTAG-AAGCTTGARGCITTYGGIAAYGCNAARAC-3' (LEAFGNAKT); 5'-ATAGAATTCATCGATTTKGGYTTNATRCACCTRATRA-3' (YIR-CIKPN); and 5'-ATAGAATTCATCGATACRTTYTCCAGCAGNCC-CAGRTA-3' (YLGLLENV). The two 5' primers contained XbaI and HindIII restriction sites 5' of the degenerate nucleotides, while the 3' primers included the EcoRI and ClaI sites. The primary templates were total RNA from PC12 cells and P6 mouse cerebellar granular neurons. The secondary template was 1–5 ml of unpurified material from the first amplification. The annealing temperature was 48°C in the first round and 54°C in the second. The remaining conditions were those recommended by the manufacturer (Perkin-Elmer Cetus, Norwalk, CT). Bovine BBMI cDNA (Hoshimaru and Nakanishi, 1987), kindly provided by Dr. S. Nakanishi (Kyoto University, Kyoto, Japan), served as the positive control. PCR products of the predicted size were purified from agarose gels (Geneclean, Bio 101, New York University, New York, NY) and directionally cloned into pGEM7Zf+ (Promega Corp., Madison, WI) at Xba I and EcoRI. Clones were analyzed by direct sequencing and by restriction digestion patterns.

cDNA Library Screening

The PCR product pGMMIα was labeled with [^{32}P]dCTP by random priming according to the manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN). This probe was used to screen an oligo-dT primed λgt11 library generated from P6 mouse cerebellar granular neurons, kindly provided by Dr. M. Hatten and Dr. S. Vidan. Clones were purified after three rounds of screening (Sambrook et al., 1989) and subcloned at NotI, SfiI sites into pGEM11Zf+ (Promega Corp.). Three unique clones were identified, λCGN74a, λCGN82a, and λCGN15, containing nucleotides 945–3998, 656–3395, and 1212–3403, respectively. By alignment to BBMI, these clones did not encode ~800 nt of the 5' end of MMIα. To obtain an overlapping clone from this region, a probe containing a novel 5' sequence was generated by PCR. The 3' primer in this PCR amplification was 5'-CCGAATTCTGAAATCCCGCTCCAGC-3', contained within the 5' region of λCGN82a. The 5' primer, 5'-ACAGAATTCAAGCTTGTNTT-IGTITCNGTNAANCC-3', was a degenerate DNA sequence of the highly conserved amino acid motif, VLVSVNP. The specific and degenerate primers were used at concentrations of 2 and 200 nM, respectively. The PCR product of the predicted size was gel purified and subcloned into pBluescript KS. Sequencing demonstrated 100% overlap with λCGN82a and high homology with BBMI in the novel 5' regions. This fragment was used to screen a λgt10 random primed adult mouse brain library, kindly provided by Dr. J. Schlessinger (New York University, New York, NY). Three clones were positive after three rounds of purification. These were subcloned into pBluescript KS at the EcoRI site and analyzed by PCR for the extent of the novel 5' sequence. One clone, λAMB9d, containing nucleotides −197–845, encompassed the remainder of the coding region.

DNA Sequencing and Analysis

The entire coding region was sequenced from both strands using the dideoxy chain-termination method (Sanger et al., 1977). Fragments were either subcloned at convenient restriction sites or digested with S1 nuclease (Erase-a-base; Promega Corp.). Junctions were sequenced by priming from complementary oligonucleotides. Contigs were assembled and sequences analyzed using the Genetics Computer Group package version 7 for the VAX (Devereux et al., 1984).

RNA Transfer Analysis

Total RNA was purified from tissues and cell lines according to the procedure of Chomczynski and Sacchi (1987). RNA transfer was performed with standard protocols (Sambrook et al., 1989). Probes were labeled by random priming with [^{32}P]dCTP as described above.

Cell Cultures and Isolation

P19 teratocarcinoma cells (McBurney and Rogers, 1982), N115 neuroblastoma cells (Schrier et al., 1974), U251 and G24-26 astrocytoma cells (Ponten, 1975; Sundarrag et al., 1975), and C17 cells (Ryder et al., 1990) were grown under standard conditions and harvested directly for the isolation of total RNA. PC12 cells (Greene and Tischler, 1976) were treated with NGF (100 ng/ml) for 5 d before harvesting. Astrocytes were purified from rat P6 cerebellum as described (Hatten, 1985).

Animals and Tissue Preparation

P0, P2, P7, P12, and adult mice were deeply anesthetized with an intraperitoneal injection of an overdose of ketacet. The mice were perfused transcardially with 0.1 M PBS, pH 7.2, prepared with diethyl pyrocarbonate-treated water, followed by 4% buffered paraformaldehyde, pH 7.2, also made with diethyl pyrocarbonate-treated water. The brains were carefully removed and placed in fixative overnight, dehydrated, and embedded in paraffin. 8-μm sections were cut and collected on silanized slides.

In Situ Hybridization

Digoxigenin-UTP-labeled cRNA probes (Genius 4 digoxigenin-UTP RNA Labeling Kit; Boehringer Mannheim Corp.) were generated from a 388-bp KpnI insert (nucleotides 2053–2440) or a XhoI to EcoRI insert (nucleotides 2850–3638), both cloned into pBluescript KS and transcribed with T3 RNA polymerase. All buffers and diluents were prepared using 0.1% diethyl pyrocarbonate-treated distilled water. Deparaffinized sections were treated with proteinase K (20 μg/ml in 10 mM Tris, 1 mM EDTA [TE]) for 25 min at room temperature. The sections were rinsed in TE, washed in 2× SSC for 10 min, and prehybridized in buffer containing 50% formamide, 20× SSC, 2 M Tris, pH 7.5, 50× Denhardt's solution, and 0.1 mg/ml salmon sperm DNA for 1 h at room temperature. Sections were incubated with the digoxigenin-UTP-labeled probe (4 μg/ml prehybridization solution) for 16 h at 42°C. The next day, sections were briefly rinsed in 2× SSC and incubated in RNAse A (200 μg/ml) in 10 mM Tris, 500 mM NaCl, and 1 mM EDTA for 15 min at 37°C. The sections were then washed with 2× SSC for 30 min at room temperature, twice in 0.5× SSC for 15 min at 42°C, once in 1× SSC for 1 h at room temperature, and briefly in 100 mM Tris and 150 mM NaCl, pH 7.5 (buffer 1). The sections were then incubated in 2% normal sheep serum with 0.3% Triton X-100 in Buffer I for 30 min at room temperature. The sections were then incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim Corp.) diluted 1:100 in buffer I with 2% normal sheep serum and 1.25% Triton X-100 overnight at 4°C. The next day the tissue was washed in buffer I three times for 10 min each, followed by a 2-min wash in 100 mM Tris, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5 (buffer III). The reaction product was visualized with 0.023% 4-nitro blue tetrazolium chloride, 0.018% 5-bromo-4-chlor-3-indolyl-phosphate (X-phosphate), and 0.024% levamisole. The blue-brown color reaction product was visible within 1–2 h. The sections were then washed in buffer IV for at least 5 min to stop the reaction. Control conditions included omission of the digoxigenin-UTP-labeled probe, labeled sense RNA, and competition with unlabeled antisense RNA. Sections were coverslipped in Aquamount, Lerner Laboratories (Pittsburgh, PA).

S1 Nuclease Protection

Probes including 46 and 51 nucleotides (MMIβ: 5'-CAAGTAGCTCTG-GGGGTTCCGTTCCAAGCCCAGACGACGAAGAGC-3', MMIγ: 5'-GACTTCAGTTGTGCCCCCACACGGATGTAGTTGTAGGATGA-GAGTGATTT-3') corresponding to stretches within pGMMIβ and pGMMIγ, respectively, that are of low homology among currently described myosin I genes, were synthesized for hybridization to total tissue RNA. S1 nuclease protection was carried out following a standard protocol as described (Greene and Struhl, 1987). Briefly, 2 nmol of the oligonucleotide probes was end labeled with [^{32}P]α-ATP by polynucleotide kinase (Boehringer Mannheim Corp.). 1.2 × 10^5 cpm for each probe was hybridized with 25 μg of total RNA overnight at 30°C. 300 U of S1 nuclease (Boehringer

Mannheim Corp.) was added for an additional hour and reactions were stopped by boiling in formamide/EDTA. Samples were analyzed on a 10% polyacrylamide/urea denaturing sequencing gel.

Results

Identification of Novel Mammalian Myosin I Genes

Both preliminary biochemical studies (Phelan et al., 1991) and the presence of multiple myosin I's in lower organisms (Pollard et al., 1991) suggested the existence of a family of mammalian myosin I genes in addition to the well-characterized BBMI. To search for such genes, we devised a two-step nested PCR strategy that was biased against amplification of conventional myosin II sequences. Two pairs of degenerate primers from conserved amino acid sequences within the myosin head domain (GAGKTEA and YLGLLEN; LEAFGN and YIRCIKP) were used in two sequential reactions. Because we sought to identify myosin I genes expressed in the nervous system, total cellular RNA from PC12 cells and postnatal day 6 mouse cerebellar granular neurons served as the templates for the first reaction. The second amplification was conducted with the nested primers using the unpurified products of the first reaction as the templates. 42 subcloned PCR products (30 from PC12 cells and 12 from granular neurons) were analyzed by restriction fragment patterns and partial sequencing. Three unique PCR products were identified and sequenced. Analysis of the nucleotide and predicted amino acid sequences confirmed that these fragments correspond to novel myosin I genes (Fig. 1 *A*). The clone derived from mouse cerebellar granular neuron RNA, designated pGMMIα, is 66 and 64% identical (based on the predicted amino acid sequence) to bovine and chicken BBMI, respectively. Over the same stretch, pGMMIα is 38% identical to cardiac muscle myosin. Of the conventional myosin sequences contained in Genbank, this myosin II gene is the most similar to pGMMIα. Clones pGMMIβ and pGMMIγ, derived from PC12 cells, are 49% identical to bovine BBMI, but only 37% homologous to the cardiac muscle myosin sequence. Additionally, there is a highly conserved stretch within all described myosin I head domains that distinguishes them from myosin II. Sequences similar to this motif, QA-yaRDALAK.iYSR (Pollard et al., 1991), are present in all three clones. Clone pGMMIγ, which diverges the most from this motif, is still 73% identical in this stretch.

Cloning and Predicted Primary Sequence of Mammalian Myosin Iα

As will be detailed below, using clone pGMMIα as a probe we detected a message with a distinctive pattern of regulation in the developing postnatal mouse brain and therefore chose this clone for further study. Screening an oligo-dT primed cDNA library from postnatal day 6 mouse cerebellar granular neurons yielded three overlapping clones ranging from 2.5 to 3.3 kb. Each of the three clones encodes a stretch of the cDNA from within the consensus myosin head through to the 3′ untranslated sequence. Two of these clones, λCGN74a and λCGN82a (see Materials and Methods), while identical in the coding region, differ in the 3′ untranslated stretch, with λCGN74a containing an additional 503 nucleotides before the polyadenylation site (Fig. 2).

To obtain a clone encoding the 5′ end, we screened a random primed mouse brain library with a PCR product that extended 5′ from the cDNA clone λCGN82a (see Materials and Methods). We isolated a 1.2-kb clone (λAMB9d) that overlapped with λCGN82a and extended 850 nucleotides to the 5′ end. These overlapping clones contain a single long open reading frame that begins with a methionine codon at the designated nucleotide 1. This sequence ACCATGG is predicted to be a strong translation initiation site (Kozak, 1989). The only in-frame methionine upstream, at position −17, would constitute a weak initiation codon. Translation of this open reading frame predicts a protein of 1,079 amino acids with a molecular mass of 125,160 D (Fig. 2).

Analysis of the predicted MMIα protein reveals three functional domains that are similar to the BBMIs: a myosin head, a calmodulin binding region, and a COOH-terminal tail with a high net positive charge. All myosin heavy chain proteins share a head domain that contains nucleotide and actin binding sites as well as other conserved residues that are probably necessary for the generation of mechanochemical force (Korn and Hammer, 1988). Analysis reveals that MMIα protein contains such a canonical myosin head (Fig. 2). A sequence that participates in ATP binding, GESGAKTE, is conserved throughout all myosins and is also present in MMIα (Fig. 2). Moreover, MMIα mRNA encodes an actin binding site, YIRCIKP(N/K), that is shared by myosin I proteins and is distinct from myosin II. These features provide further evidence that MMIα encodes a member of the myosin I family.

Immediately COOH terminal to the head domain, the predicted MMIα protein appears to encode, by comparison to BBMI, a series of calmodulin binding sites. Calmodulin serves as the light chain for BBMIs and other unconventional vertebrate myosins (Mooseker and Coleman, 1989; Espreafico et al., 1992), and its interaction with Ca^{2+} has been shown to regulate ATPase activity and in vitro motility (Mooseker et al., 1989; Collins et al., 1990). This region was mapped in the chick BBMI by α-chymotryptic mapping and construction of fusion proteins with calmodulin binding capacity (Coluccio and Bretscher, 1988; Halsall and Hammer, 1990). Comparison of this domain in BBMI with the corresponding region in the MMIα protein shows an amino acid identity of 56% and, with conservative substitutions, a sequence similarity of 74%. Prior reports evaluating this domain in BBMI have based estimates of the number of sites on an alignment of this region with the well-defined calmodulin binding domain of neuromodulin (Swanljung-Collins and Collins, 1992), and with a similar motif in neurogranin (Baudier et al., 1991). This comparison was based on the observation that both BBMI and neuromodulin bind calmodulin in the absence of Ca^{2+} (Coluccio and Bretscher, 1987; Alexander et al., 1988). A similar comparison of this region in MMIα reveals five potential calmodulin binding sites (Fig. 2). There are conserved aromatic or aliphatic residues at positions 1, 5, and 8, as well as basic amino acids clustered in the COOH-terminal part of these sites.

The alignment of the calmodulin binding region also reveals a 29 amino acid insert within MMIα that is absent in bovine BBMI. An insert, similar in both length and relative position within the message, was recently reported as a rare splice variant of chicken BBMI (Fig. 2) (Halsall and Hammer, 1990). A synthetic peptide of this fragment was shown

A

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MMIα      LEAFGNAKTV RNDNSSRFGK YMDIEFDFKG DPLGGVISNY LLEKSRVVKQ PRGERNFHVF YQLLSGASEE LLYKLKLERD  228
MMIβ      LEAFGNAKTL RNDNSSRFGK YMDVQFDFKG APVGGHILSY LLEKSRVVHQ NHGERNFHVF YQLLEGGEEE ALRRLGLERN   80
MMIγ      LEAFGNAKTN RNDNSSRFGK YMDINFDFKG DPIGGHINNY LLEKSRVIVQ QPGERSFHSF YQLLQGGSEQ MLHSLHLQKS   80
BovBBMI   LEAFGNAKTI RNNNSSRFGK YMDIEFDFKG FPLGGVITNY LLEKSRVVKQ LEGERNFHIF YQLLAGADAQ LLKALKLERD  221
ChkBBMI   LEAFGNAKTI RNDNSSRFGK YMDVEFDFKG DPLGGVISNY LLEKSRIVRH VKGERNFHIF YQLLAGGSAQ LLQQLKLRPD  179
AcaMIb    LEAFGNAKTL LNNNSSRFGK YFEIHFNRLG EPCGGRITNY LLEKSRVTFQ TRGERSFHIF YQLLAGASDA EAQEMQLY.A  218
CrdMII    LEAFGNAKTV RNDNSSRFGK FIRIHFGATG KLASADIETY LLEKSRVIFQ LKAERNYHIF YQILSNKKPE LLDMLLVTNN  306

MMIα      FSRYNYLSLD S.AKVNGVDD AANFRTVRNA MQIVGFLDHE AEAVLEVVAA VLKLGNIEFK PESRVNGLDE SKIKDKIELN  307
MMIβ      PQSYLYLVKG QFAKVSSIND KSDWKLLRKA LSVIDFTEDE VEDLLSIVAS VLHLGNIHFA ADEDSNAQVT TE.....NQL  155
MMIγ      LSSYNYIRVG A.QLKSSIND AAEFKVVADA MKVIGFKPEE IQTVYKILAA ILHLGNLKFI VDGDTPLIEN GKVVSV....  155
BovBBMI   TGGYAYLNPD T.SRVDGMDD DANFKVLQSA MTVIGFSDEE IRQVLEVAAL VLKLGNVELI NEFQANGVPA SGIRDGRGVQ  300
ChkBBMI   CSHYGYLNHE K.SVLPGMDD AANFRAMQDA MAIIGFAPAE VTALLEVTAV VLKLGNVKLS SSFQASGMEA SSIAEPRELQ  258
AcaMIb    PENFNYLNQS ACYTVDGIDD IKEFADTRNA INVMGMTAEE QRQVFHLVAG ILHLGNVAFH DGGKGT.... ..........  284
CrdMII    PYDYAFVSQG EVS.VASIDD SEELLATDSA FDVLGFTAEE KAGVYKLTGA IMHYGNMKFK QKQREEQAEP DGTEDA....  381

MMIα      EKFASRPASV KVVLERAFSF RTVEAKREKV ST......TL NVAQAYYARD ALAKNLYSRL FSWLVNRINE SIKAQ.....  376
MMIβ      KYLTRLLGVE GTTLREALTH RKIIAKGEEL LS......PL NLEQAAYARD ALAKAVYSAT FTWLVRKINR SVPAKDAESP  229
MMIγ      ..IAELLSTK ADMVEKALLY RTVATARDII DK......QH TEQEASYGRD AFAKAIYERL FGWIVTRIND IIAVKNYDTT  227
BovBBMI   E.IGELVGLN SVELERALCS RTMETAKEKV VT......TL NVIQAQYARD ALAKNIYSRL FNWLVNRINE SIKVG.....  368
ChkBBMI   E.ISQLIGLD PSTLEQALCS RTVKVRDESV LT......AL SVSQGYYGRD ALAKNIYSRL FDWLVNRINT SIQVK.....  326
AcaMIb    ...AAVHDRT PFALKNALLF RVLNTGGAGA KKMSTYNVPQ NVEQAASARD ALAKTIYSRM FDWIVSKVNE ALQKQ..GGS  359
CrdMII    DKSAYLMGLN SADLLKGLCH PRVKVGNEYV TK......GQ SVQQVYYSIG ALAKSVYEKM FNWMVTRINA TLETK.....  450

MMIα      TKVRKKVMGV LDIYGFEIFE DNSFEQFIIN YCNEKLQQIF IELTLKEEQE EYIREDIEWT HIDY.FNNAI ICDLIENNT.  454
MMIβ      SWRSTTVLGL LDIYGFEVFQ HNSFEQFCIN YCNEKLRQLF IELTLKSEQE EYEAEGIAWE PVQY.FNNKI ICDLVEEKF.  307
MMIγ      VHGKNTVIGV LDIYGFEIFD NNSFEQFCIN YCNEKLQQLF IQLVLKQEQE EYQREGIPWK HIDY.FNNQI IVDLVEQQH.  305
BovBBMI   TGEKRKVMGV LDIYGFEILE DNSFEQFVIN YCNEKLQQVF IEMTLKEEQE EYKREGIPWV KVEY.FDNGI ICNLIEHNQ.  446
ChkBBMI   PGKQRKVMGV LDIYGFEIFQ DNGFEQFIIN YCNEKLQQIF ILMTLKEEQE EYVREAIQWT PVEF.FDNSI ICDLIENSK.  404
AcaMIb    GDHNNNMIGV LDIFGFEIFE QNGFEQFCIN YVNEKLQQYF IELTLKAEQE EYVNEGIQWT PIKY.FNNKV VCELIEGKRP  438
CrdMII    .QPRQYFIGV LDIAGFEIFD FNSFEQLCIN FTNEKLQQFF NHHMFVLEQE EYKKEGIEWE FIDFGMDLQA CIDLIE..KP  527

MMIα      NGILAMLDEE CLR...PGTV TDETFLEKL. NQVCATHQHF ESRMSKCSRF LNDTTLPHSC FRIQHYAGKV LYQVEGFVDK  530
MMIβ      KGIISILDEE CLR...PGEA TDLTFLEKL. EDTIKHHPHF LTHKL..ADQ KTRKSLDRGE FRLLHYAGEV TYSVTGFLDK  381
MMIγ      KGIIAILDDA CMN...VGKV TDGMFLEAL. NSKLGKHGHF SSRKTCASDK ILEFDRD... FRIRHYAGDV VYSVIGFIDK  378
BovBBMI   RGILAMLDEE CLR...PGVV SDSTFLAKL. NQLFSKHSHY ESKVTQNAQR QYDHSMGLSC FRICHYAGKV TYNVNSFIDK  522
ChkBBMI   VGILAMLDEE CLR...PGTV NEDTFITKL. NQIFASHKRY ESKETLNAKH VTDVSLPLRC FRIHHYAGKV TYNVTGFIEK  480
AcaMIb    PGIFSLLDDI CFTMHAQSDG MDGKFLQKC. QGGFPSHLHF RGMN..... ........NA FSIKHYAGEV TYEAEGFCEK  503
CrdMII    MGIMSILEEE CMF....PKA TDMTFKAKLY DNHLGKSNNF QKPRNVKGKQ EAH....... FSLVHYAGTV DYNILGWLEK  596

MMIα      NNDLLYRDLS QAMWKADHSL IKSLFP.... ..EGNPAKVN L.KRP....P TAGSQFKASV ATLMRNLQTK NPNYIRCIKP  599
MMIβ      NNDLLFRNLK ETMCSSTNPI MAQCFD.... ..KSELSD.. .KKRP....E TVATQFKMGL LQLVEILRSK EPAYIRCIKP  448
MMIγ      NKDTLFQDFK RLMYNSSNPV LKNMWP.... ..EGKLSITE VTKRP....L TAATLFKNS. .......... ..........  427
BovBBMI   NNDLLFRDLS QAMWKARHPL LRSLFP.... ..EGDPKQAS L.KRP....P TAGAQFKSSV TTLMKNLYSK NPNYIRCIKP  591
ChkBBMI   NNDLLFRDLS QAMWAARHTL LRSLFP.... ..EGDPQRPS L.KLP....P TTGSQFKASV ATLMKNLYSK NPNYIRCIKP  549
AcaMIb    NKDTLFDDLI AVIQESENRL LVSWFP.... ..EDTKQLQ. .KKRP....T TAGFKLKTSC DALMEALSRC SPHYIRCIKP  571
CrdMII    NKDPLNETVV GLYQKSSLKL MATLFSTYAS ADTGDSGKGK GGKKKGSSFQ TVSALHRENL NKLMTNLRTT HPHFVRCIIP  676
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B

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MMIα      .SELFKDKKA LYPSSVGQPF QGAYLEINKN PKYKKLKDAI EEKIIIAEVV NKINRANGKS TSRIFLLTNN NLLLADQKSG  966
BovBBMI   ASELFKGKKA SYPQSVPIPF HGDYIGLQRN PKLQKLKGGE EGPILMAETV VKVNRGNAKT SSRILLLTKG HVIITDMKNP  929
ChkBBMI   ..QLFKDKKA LYAQSLQQPF RGEYLGLTQN RKYQKLQAVA KDKLVMAEAV QKVNRANGKT VPRLLLLTTE HLVLADPKAA  887
Consensus .seLFKdKKA lYp-Sv.qPF .G.Yl--..N pKy-KLk... e.ki.-AE.V .K-NRaNgK- .sRi-LLT.. -l.laD.K..

MMIα      QIKSEVPLVD VTKVSMSSQN DGFFAVHLKE GSEAASKGDF LFSSDHLIEM ATKLYRTTLS QTKQKLNIEI SDEFLVQFRQ 1046
BovBBMI   QAKTVIPLNS LAGVSVTSFK DGLFSLHLSE ISSVGSKGEF LLVSEHVIEL LTKICRATLD ATQMQLPVTV TEEFSVKFKE 1009
ChkBBMI   QPKMVLSLCD IQGASVSRFS DGLLALHLKE TSTAGGKGDL LLVSPHLIEL VTRLHQTLMD ATAQALPLSI ADQFSTRFPK  967
Consensus Q.K.-.pL.d ..-vS-ss-. DG-fa-HLkE .S.a-sKGdf L--S.HlIE- .Tkl.rttl- -T.q.Lp..i .deF-v.F..

MMIα      DKVCVKFIQG NQKNGSVPTC .KRKNNRLLE VAVP*  1080
BovBBMI   GSLTVKVIQG PGGGGTGKLS FKKKGSRCLE VTVQ*  1044
ChkBBMI   GDVAVTVVES AKGGGDVPVC .KKRGSHKME ILVH*  1001
Consensus -.v.Vk-iqg ..--G.vp.c .K-k--r.lE v.V.*
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Figure 1. Alignment of myosin I head and tail sequences. (*A*) Predicted protein sequences for MMIα, MMIβ, MMIγ, and other myosin protein sequences were aligned within the overlapping head domain using the Pileup program (Devereux et al., 1984) of the GCG package. Where applicable, numbers indicate the amino acid residue according to published sequences. The residue numbers for MMIβ and MMIγ start at the 5′ ends of the PCR products. The sequences are not listed in the order of their relatedness. MMIγ does not extend to the 3′ end because this PCR product was cut at a HindIII site at serine 427 in preparation for subcloning. Examples of residues and domains that are highly conserved in the myosin I but not in the myosin II gene family are underlined. *BovBBMI*, bovine BBMI (Hoshimaru and

to bind calmodulin, also independent of Ca^{2+} concentration.

The COOH-terminal, or tail, region of BBMIs and an equivalent region in invertebrate myosin I's have a high net positive charge and have been shown to bind with high affinity to negatively charged phospholipids (Adams and Pollard, 1989). This interaction has been proposed as a means by which myosin I's associate with membranes in vivo (Miyata et al., 1989; Hayden et al., 1990; Doberstein and Pollard, 1992; Zot et al., 1992). The predicted protein for MMIα also contains a COOH-terminal region with a high net positive charge and a predicted pI of 10.25. In comparison, the pI's of the equivalent regions in chick and bovine BBMI are 10.41 and 10.44, respectively. The similarity within this region extends beyond a simple summation of charge. Alignment of this region with those of chicken and bovine myosin I reveals conserved amino acid stretches that may reflect shared functional domains (Fig. 1 *B*).

There are also major structural differences between MMIα and most of the myosin I's identified in Acanthamoeba and Dictyostelium. Unlike these myosins, yet similar to BBMIs and the Dictyostelium actin-based motor A (Titus et al., 1989), MMIα does not encode an ATP-independent actin binding site (Pollard et al., 1991).

Expression Pattern of MMIα, MMIβ, and MMIγ mRNAs

Northern blot analysis of the expression of MMIα mRNAs in various adult murine tissues reveals messages of 4.1, 4.9, and 6.5 kb that are most prominently detected in the brain, lung, and liver (Fig. 3). These messages are also expressed in the heart and testes, but are not detectable or are expressed at very low levels in the kidney, small intestine, skeletal muscle, adrenal gland, or spleen (data not shown). MMIα mRNA also shows variable expression in cultured cells and established cell lines. All three messages are present in the mouse neuroblastoma N115 line, the neural precursor line C17, and the murine teratocarcinoma cell line P19, yet are not detectable in the pheochromocytoma-derived PC12 cell line. MMIα transcripts are not detectable in the astrocytic cell lines U251 and G26-24, nor in isolated astrocytes from P6 rat cerebellum (Hatten, 1985) (data not shown). All three messages are present in normal rat kidney fibroblasts and NIH-3T3 cells, while in the CHO cell line only the 4.1-kb message is detected (data not shown).

The three MMIα messages noted above are detected by probes representing stretches of the coding region of the gene. To evaluate the correspondence of the detected messages with the isolated cDNAs, a subcloned fragment from the 3′ untranslated region of clone λCGN74a that is not contained within clone λCGN82a (see above) was used to probe total RNA from various murine tissues. As illustrated in Fig. 3 *A* (lane 7), this probe detects the 4.9- and 6.5-kb messages but not the 4.1-kb message. This indicates that alternate sites of polyadenylation appear to account for the size difference between the two smaller messages. These findings also demonstrate that the 6.5-kb message contains the longer 3′ untranslated segment, but the additional modifications necessary to generate this message are unknown.

MMIβ and MMIγ display patterns of mRNA expression that are different both from each other and from MMIα. In PC12 cells, a labeled pGMMIβ probe hybridizes to a single 5.0-kb message. This band is detected in all tissues and cell lines that express MMIβ mRNA. In tissue an additional 6.9-kb message is also detected with pGMMIβ. The pGMMIγ probe also detects a complex pattern of mRNAs, even at high stringency (0.2× SSC, 60°C). Hybridization to at least two messages of 5.2 and 7.0 kb is observed, although, as for MMIβ, in PC12 cells only the 5.2-kb band is detected. The tissue and cell line distributions of MMIβ and MMIγ mRNAs are distinct. MMIβ message is widely expressed: it is found in adult lung, kidney, heart, small intestine, adrenal gland, skeletal muscle, and P2 cerebral cortex (Fig. 4), as well as testes, spinal cord, and the adult cerebral cortex (data not shown). The 5.0-kb MMIβ message is also present in numerous cell lines of neural, glial, and fibroblastic origin (Fig. 4 *B*). MMIγ mRNA, though not as widely expressed as MMIβ, is found in lung, kidney, small intestine, and adrenal gland, and in the adult cerebral cortex and spinal cord (data not shown). MMIγ is present at relatively low levels in the liver, heart, skeletal muscle, and P2 cerebral cortex. It is also not detected in several cell lines in which MMIβ is found: NIH3T3, U251-1, and P19 cells.

S1 nuclease analysis was performed to provide an additional assessment of the tissue distribution of MMIβ and MMIγ mRNA, as well as to rule out possible crossreactions in Northern blots. Probes were chosen from regions of pGMMIβ and pGMMIγ with very low homology to each other or to MMIα (see Materials and Methods). Bands corresponding to the predicted protected fragment sizes were detected after S1 nuclease digestion of RNA from lung, liver, kidney, skeletal muscle, heart, and small intestine. Furthermore, we did not detect protected fragments of smaller sizes that would suggest crossreaction with a homologous transcript. For both clones, the relative levels of detected signal approximately corresponded to the relative abundance of mRNAs detected by Northern blot analysis with the exception of liver RNA, which had a higher signal by S1 nuclease protection (data not shown).

Developmental Expression of MMIα mRNA in Neurons of the Postnatal Mouse Brain

Examination of MMIα mRNA in the postnatal mouse brain by Northern blot analysis points to a developmental regulation of the steady-state levels for these messages. As shown in Fig. 3 *B*, all three messages reach a peak at postnatal day 2 (P2) and decline thereafter to relatively low levels in the adult (Fig. 3, *A* and *B*). In the cerebellum, a peak of expres-

Nakanishi, 1987); *ChkBBMI*, chicken BBMI (Garcia et al., 1989); *AcaMIb*, Acanthamoeba myosin Ib gene (Jung et al., 1987); *CrdMII*, the rat alpha cardiac muscle myosin II (McNally et al., 1989). (*B*) Alignment of the tail sequences of MMIα protein and both chicken and cow BBMI. In the consensus, a residue is listed as a capital letter if shared by all three sequences, a lowercase letter if shared by MMIα and one other sequence, a dash if shared by both BBMI, and a period if different in all three.

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GGACTGGGAAAAATGACTCTCATAAATTACATAAAGAGGGTTCCTTGGCCAATGTTGCTACTACTTCATTTGAATTGCTTCCTTTGAAGTAAGTTTCCTTTCGGGATGACCTCCTGGCCT -76
TTGTGGATATGTGCAGTGAAGTTCTGAGAAGGAGCTGTGGCTGTCACACATCTGCCtGATGCCTCCGCTGGAAACC  0

ATG GCC AAA ATG GAG GTG AAA TCC TCA CTT CTG GAC AAT ATG ATC GGT GTT GGA GAT ATG GTT CTT TTA GAG CCG CTC AAT GAG GAG ACG  90
Met Ala Lys Met Glu Val Lys Ser Ser Leu Leu Asp Asn Met Ile Gly Val Gly Asp Met Val Leu Leu Glu Pro Leu Asn Glu Glu Thr  30

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Phe Ile Asp Asn Leu Lys Lys Arg Phe Asp His Asn Glu Ile Tyr Thr Tyr Ile Gly Ser Val Val Ile Ser Val Asn Pro Tyr Arg Ser  60

TTG CCC ATA TAT TCG CCG GAG AAA GTG GAA GAT TAC AGA AAT AGA AAC TTC TAT GAA CTG AGC CCT CAC ATC TTT GCT TTG TCG GAT GAA  270
Leu Pro Ile Tyr Ser Pro Glu Lys Val Glu Asp Tyr Arg Asn Arg Asn Phe Tyr Glu Leu Ser Pro His Ile Phe Ala Leu Ser Asp Glu  90

GCA TAC AGA TCC CTC CGT GAT CAA GAT AAA GAT CAA TGC ATT CTC ATT ACT GGG GAG AGT GGA GCA GGA AAA ACA GAG GCC AGC AAG CTC  360
Ala Tyr Arg Ser Leu Arg Asp Gln Asp Lys Asp Gln Cys Ile Leu Ile Thr Gly Glu Ser Gly Ala Gly Lys Thr Glu Ala Ser Lys Leu  120

GTC ATG TCC TAT GTC GCT GCC GTG TGC GGA AAA GGA GCA GAA GTC AAT CAG GTC AAG GAA CAG CTT CTG CAG TCC AAC CCT GTC CTG GAA  450
Val Met Ser Tyr Val Ala Ala Val Cys Gly Lys Gly Ala Glu Val Asn Gln Val Lys Glu Gln Leu Leu Gln Ser Asn Pro Val Leu Glu  150

GCT TTT GGA AAT GCC AAG ACT GTG AGG AAT GAC AAC TCA TCT AGA TTC GGC AAA TAC ATG GAT ATT GAA TTT GAC TTT AAA GGC GAT CCG  540
Ala Phe Gly Asn Ala Lys Thr Val Arg Asn Asp Asn Ser Ser Arg Phe Gly Lys Tyr Met Asp Ile Glu Phe Asp Phe Lys Gly Asp Pro  180

CTG GGA GGA GTG ATA AGT AAC TAT CTT TTA GAG AAA TCT CGT GTT GTT AAG CAG CCA AGA GGT GAA AGA AAC TTC CAT GTG TTC TAT CAG  630
Leu Gly Gly Val Ile Ser Asn Tyr Leu Leu Glu Lys Ser Arg Val Val Lys Gln Pro Arg Gly Glu Arg Asn Phe His Val Phe Tyr Gln  210

CTG CTC TCA GGC GCC TCT GAG GAG CTC CTC TAC AAG CTT AAG CTG GAG CGG GAT TTC AGC AGA TAC AAC TAT CTG AGT CTG GAC TCT GCT  720
Leu Leu Ser Gly Ala Ser Glu Glu Leu Leu Tyr Lys Leu Lys Leu Glu Arg Asp Phe Ser Arg Tyr Asn Tyr Leu Ser Leu Asp Ser Ala  240

AAA GTT AAT GGT GTG GAT GAT GCT GCC AAT TTC AGA ACT GTT AGG AAT GCA ATG CAG ATC GTG GGC TTT CTG GAT CAT GAG GCC GAG GCT  810
Lys Val Asn Gly Val Asp Asp Ala Ala Asn Phe Arg Thr Val Arg Asn Ala Met Gln Ile Val Gly Phe Leu Asp His Glu Ala Glu Ala  270

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Val Leu Glu Val Val Ala Ala Val Leu Lys Leu Gly Asn Ile Glu Phe Lys Pro Glu Ser Arg Val Asn Gly Leu Asp Glu Ser Lys Ile  300

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Lys Asp Lys Ile Glu Leu Asn Glu Lys Phe Ala Ser Arg Pro Ala Ser Val Lys Val Val Leu Glu Arg Ala Phe Ser Phe Arg Thr Val  330

GAA GCG AAG AGG GAG AAA GTT TCA ACC ACG CTG AAT GTG GCT CAG GCT TAT TAT GCC CGT GAT GCC CTG GCT AAA AAC CTC TAC AGC CGC 1080
Glu Ala Lys Arg Glu Lys Val Ser Thr Thr Leu Asn Val Ala Gln Ala Tyr Tyr Ala Arg Asp Ala Leu Ala Lys Asn Leu Tyr Ser Arg  360

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GGC TTT GAA ATA TTT GAG GAT AAC AGC TTC GAG CAG TTT ATT ATT AAC TAT TGT AAT GAA AAG CTT CAA CAA ATC TTC ATC GAA CTT ACC 1260
Gly Phe Glu Ile Phe Glu Asp Asn Ser Phe Glu Gln Phe Ile Ile Asn Tyr Cys Asn Glu Lys Leu Gln Gln Ile Phe Ile Glu Leu Thr  420

CTC AAA GAA GAG CAA GAG GAA TAT ATT CGG GAG GAC ATA GAA TGG ACT CAC ATT GAC TAC TTC AAC AAT GCT ATC ATT TGT GAC CTA ATA 1350
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AAC CAA GTC TGT GCC ACC CAC CAG CAC TTT GAG AGC AGG ATG AGC AAG TGT TCC CGC TTT CTC AAC GAC ACA ACC CTG CCC CAC AGC TGC 1530
Asn Gln Val Cys Ala Thr His Gln His Phe Glu Ser Arg Met Ser Lys Cys Ser Arg Phe Leu Asn Asp Thr Thr Leu Pro His Ser Cys  510

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Phe Arg Ile Gln His Tyr Ala Gly Lys Val Leu Tyr Gln Val Glu Gly Phe Val Asp Lys Asn Asn Asp Leu Leu Tyr Arg Asp Leu Ser  540

CAA GCC ATG TGG AAG GCC GAC CAC TCC CTC ATC AAG TCT CTG TTT CCT GAA GGA AAT CCT GCA AAG GTC AAC CTG AAG AGA CCT CCC ACC 1710
Gln Ala Met Trp Lys Ala Asp His Ser Leu Ile Lys Ser Leu Phe Pro Glu Gly Asn Pro Ala Lys Val Asn Leu Lys Arg Pro Pro Thr  570

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GCA GGC TAT GCT TTC AGG CAG GCC TAT GAA CCT TGC CTG GAA AGA TAC AAG ATG CTT TGC AAA CAA ACG TGG CCT CAC TGG AAG GGA CCA 1980
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CCA CGG ACA TTA TTC CAA CTA GAA GAC CTA AGG AAG CAG CGG CTG GAG GAC CTG GCC ACT CTC ATT CAG AAG ATT TAT CGA GGA TGG AAA 2160
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TGC CGT ACA CAT TTT CTG CTC ATG AAA AGA AGC CAA GTT GTG ATT GCT GCC TGG TAC AGA CGA TAT GCG CAA CAA AAG CGG TAC CAG CAG 2250
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Lys Ile Tyr Glu Phe Thr Leu Gln Arg Ile Val Gln Lys Tyr Leu Leu Glu Met Lys Asn Lys Met Pro Ser Leu Ser Pro Ile Asp Lys  840

AAT TGG CCA TCA AGA CCT TAC CTG TTC TTG GAT TCG ACT CAC AAG GAG CTA AAA AGG ATC TTC CAC TTG TGG AGG TGT AAA AAA TAC AGG 2610
Asn Trp Pro Ser Arg Pro Tyr Leu Phe Leu Asp Ser Thr His Lys Glu Leu Lys Arg Ile Phe His Leu Trp Arg Cys Lys Lys Tyr Arg  870

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Ser Val Gly Gln Pro Phe Gln Gly Ala Tyr Leu Glu Ile Asn Lys Asn Pro Lys Tyr Lys Lys Leu Lys Asp Ala Ile Glu Glu Lys Ile  930

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Tyr Arg Thr Thr Leu Ser Gln Thr Lys Gln Lys Leu Asn Ile Glu Ile Ser Asp Glu Phe Leu Val Gln Phe Arg Gln Asp Lys Val Cys 1050

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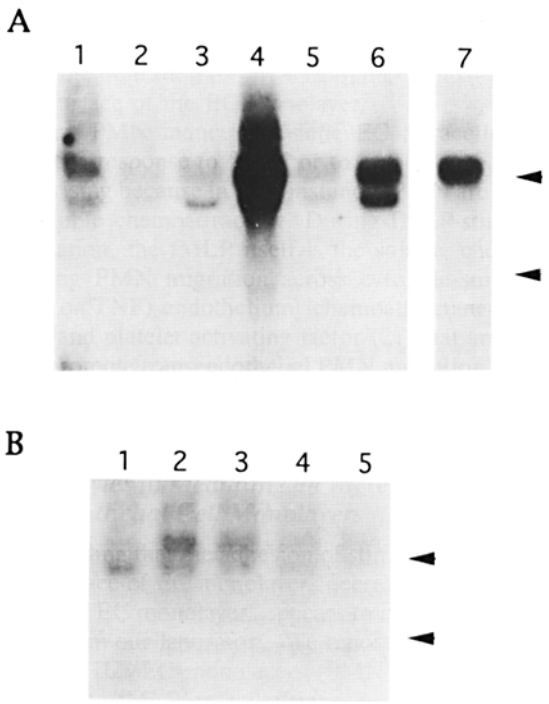

Figure 3. Tissue distribution and development regulation of MMIα mRNAs. 15 μg of total RNA from murine tissues or cell lines was analyzed by Northern blot hybridization with a 789-bp XhoI-EcoRI probe including amino acids 950–1079, except for the sample in lane 7. Unless indicated, tissue is from adult animals. 28S and 18S rRNAs are indicated by arrowheads. (*A*) Lane *1*, P2 cerebral cortex; lane *2*, adult cerebral cortex; lane *3*, N115 neuroblastoma cell line; lane *4*, lung; lane *5*, testes; lane *6*, liver; lane *7*, lung. The RNA in lane *7* was hybridized with a probe containing only 3′ untranslated nucleotides 3638–3998 that are contained within clone λCGN74a and not clone λCGN82a. (*B*) Total RNA from developing mouse cerebral cortex. Lane *1*, P0; lane *2*, P2; lane *3*, P6; lane *4*, P10; lane *5*, adult.

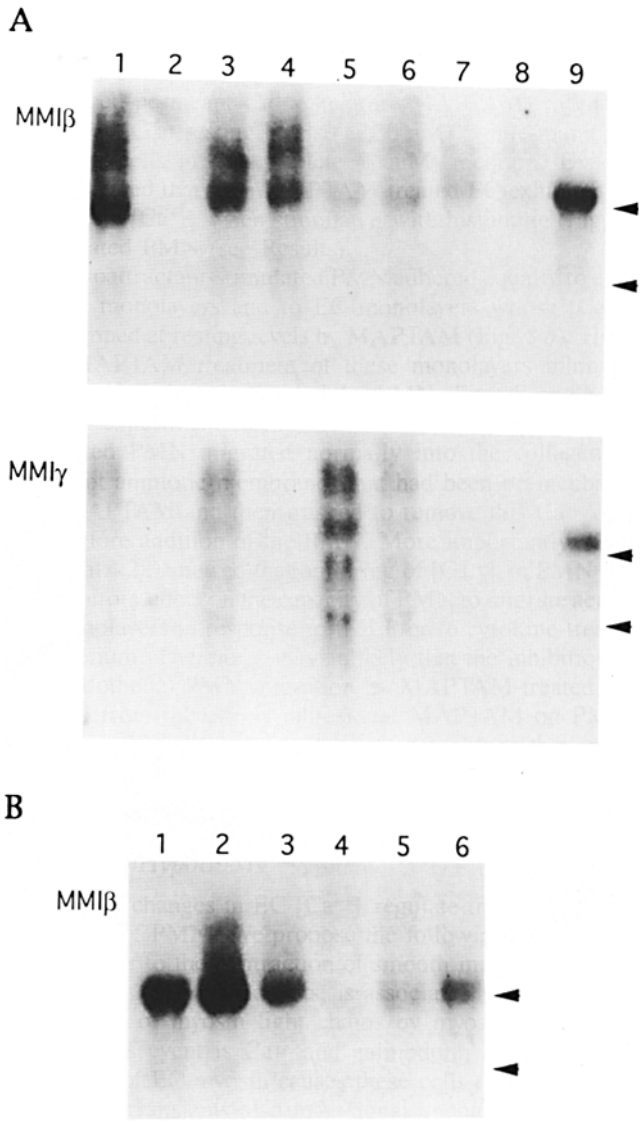

Figure 4. Tissue and cell line distribution of MMIβ and MMIγ. 10 μg of total RNA from rat tissue or cell lines was analyzed by Northern blot hybridization with probes from the indicated 1.2-kb PCR product. The positions of 28S and 18S rRNA are denoted by arrowheads. (*A*) Lane *1*, lung; lane *2*, liver; lane *3*, kidney; lane *4*, heart; lane *5*, small intestine; lane *6*, adrenal gland; lane *7*, hind leg skeletal muscle; lane *8*, P2 cerebral cortex; lane *9*, PC12 cells. (*B*) Lane *1*, PC12 cells; lane *2*, NIH 3T3 cells; lane *3*, U252-1 astrocytoma; lane *4*, C17 cells; lane *5*, P19 cells; lane *6*, P19 cells + 5 μM retinoic acid.

sion occurs at P6, whereas the amount of message present in the adult is below detection by this approach (data not shown).

To provide further information regarding the cellular, regional, and developmental distribution of MMIα mRNA in the brain, we carried out in situ hybridization. Paraffin sections of brain tissue from mice ages P0 through adult were hybridized with a digoxigenin-labeled 400-nt cRNA probe containing both head and calmodulin domain sequence. Identical staining patterns are seen with an 800-nt probe against tail sequence. No staining is observed in the presence of excess unlabeled probe or with sense probes (data not shown).

In adult mice, MMIα mRNA is widely distributed in neurons throughout the brain. Regions examined included the cerebral cortex, hippocampus, and dentate gyrus, subcortical areas, brainstem, and cerebellum. All stained cells contain large nuclei and a thin ring of cytoplasm (Fig. 5 *b*), characteristic of neurons; an extensive search did not reveal reaction product in any cells of glial or astrocytic morphology. However, reaction product is found in cells of the choroid plexus. Comparison with adjacent sections stained

Figure 2. Nucleotide and predicted amino acid sequence of MMIα cDNA. Full-length MMIα cDNA was assembled from overlapping clones λAM9d, λCGN82a, λCGN15, and λCGN74a. Conserved stretches within the ATP (amino acids 108–115) and actin binding (amino acids 593–600) sites are indicated by double underlining. The five potential calmodulin binding domains spanning amino acids 712–875 are underlined. The 29 amino acid insert within this region includes residues 767–795. The two double underlined adenines at nucleotide positions 3395 and 3998 are potential polyadenylation sites as deduced from analysis of clones λCNG82a and λCGN74a.

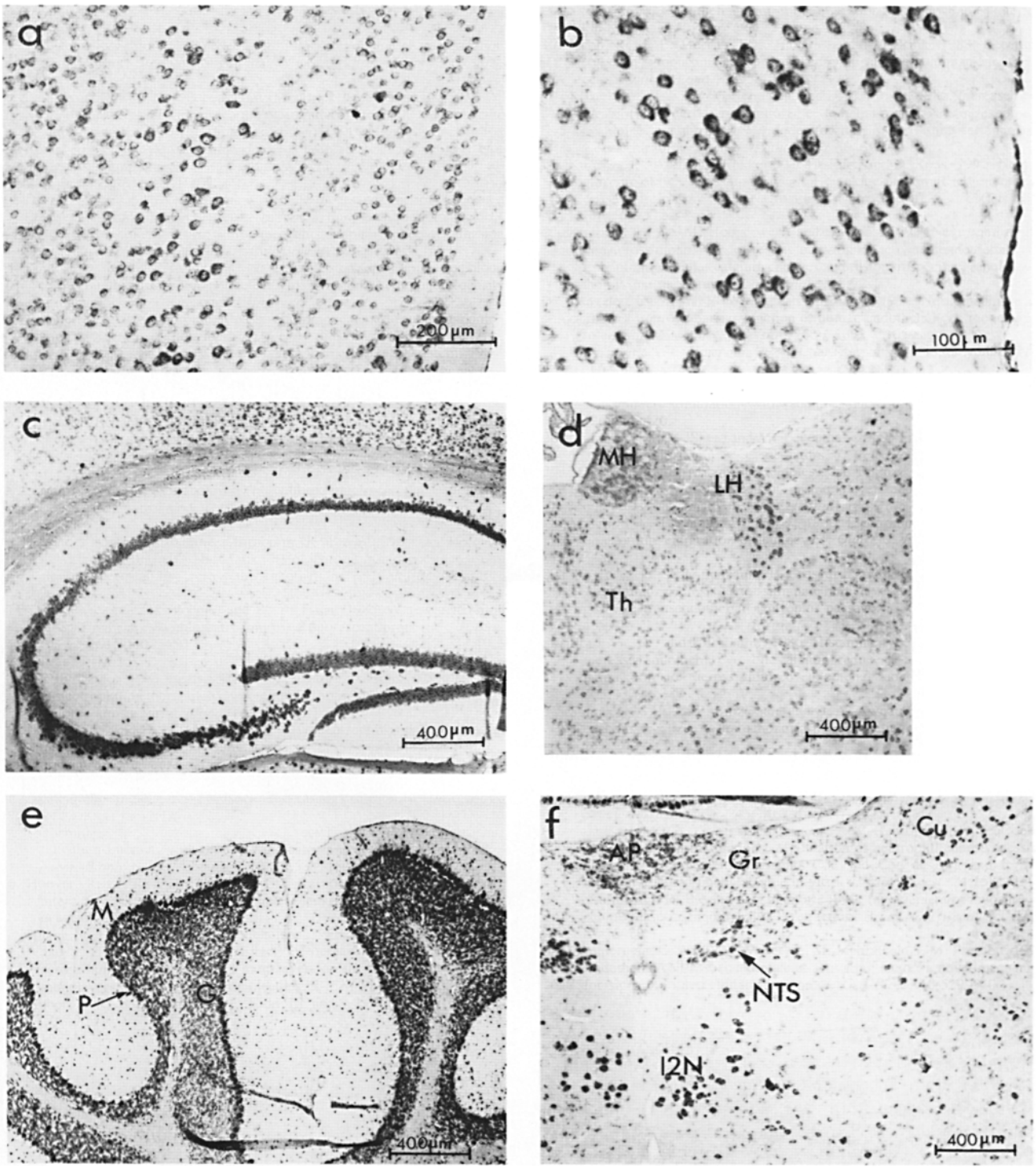

Figure 5. Photomicrographs of in situ localization of MMIα mRNA in adult mouse brain. These coronal sections from an adult mouse depict the in situ localization of MMIα mRNA in the cerebral cortex (*a*), high magnification micrograph of the cerebral cortex (*b*), hippocampus (*c*), subcortical diencephalon (*d*), cerebellum (*e*), and brainstem (*f*). The color reaction in the section of the cerebellum was allowed to develop longer in order to enhance the labeling intensity of the granular layer. *MH*, medial habenula; *LH*, lateral habenula; *Th*, thalamus; *M*, molecular layer; *p*, Purkinje cell layer; *G*, granular layer; *AP*, area postrema; *NTS*, nucleus of the solitary tract; *12N*, hypoglossal nucleus; *Gr*, nucleus gracilis; *Cu*, nucleus cuneatus.

with hematoxylin and eosin (data not shown) reveals that nearly all neurons appeared labeled, although the intensity of staining showed regional differences. For instance, in the cerebral cortex, the intensity of reaction product is greater in neurons of layer three than in neurons of the other cortical layers (Fig. 5 *a*). Also, in the cerebellum Purkinje cells and neurons of the deep nuclei stain much more intensely for MMIα mRNA than do granule neurons. In contrast, labeling in the hippocampus and dentate gyrus appears to be uniformly strong in all neuron layers.

Examination of P0, P2, P7, and P12 brains reveals that the proportion of positively stained neurons does not vary significantly during central nervous system maturation. However, there are both regional and temporal changes in expression of MMIα mRNA during development. In the hippocampus a uniformly intense level of staining is detected from P0 through adult. In areas including the cerebral cortex and cerebellum, however, the overall level of staining declines with development. For example, in consonance with Northern blot data, neurons within the cerebral cortex generally show peak levels of expression between days P2 and P7. In cerebellum at day P2, granular neurons exhibit intense and uniform labeling, whereas neurons in the Purkinje layer do not stain uniformly. This situation progressively changes with development so that, by P12 and onward, staining in Purkinje cells is uniformly intense and a relatively light staining pattern is observed in all cerebellar granular neurons (Fig. 6).

Hybridization analysis also indicates expression of MMIα mRNA in at least certain neuronal precursors. In the developing mouse cerebellum, neuroblasts migrate to form the external granular (or germinal) layer (EGL) where they continue to divide postnatally and give rise to granule, stellate, and basket cells (Jacobson, 1991). Throughout the developmental period examined (P0 to P12) all cells in the EGL label with probes to MMIα. However, staining was more intense in cells of the superficial layers of the EGL (Fig. 6). It is in this region of the EGL where granule cells continue to undergo mitosis and are just beginning to extend their bipolar processes.

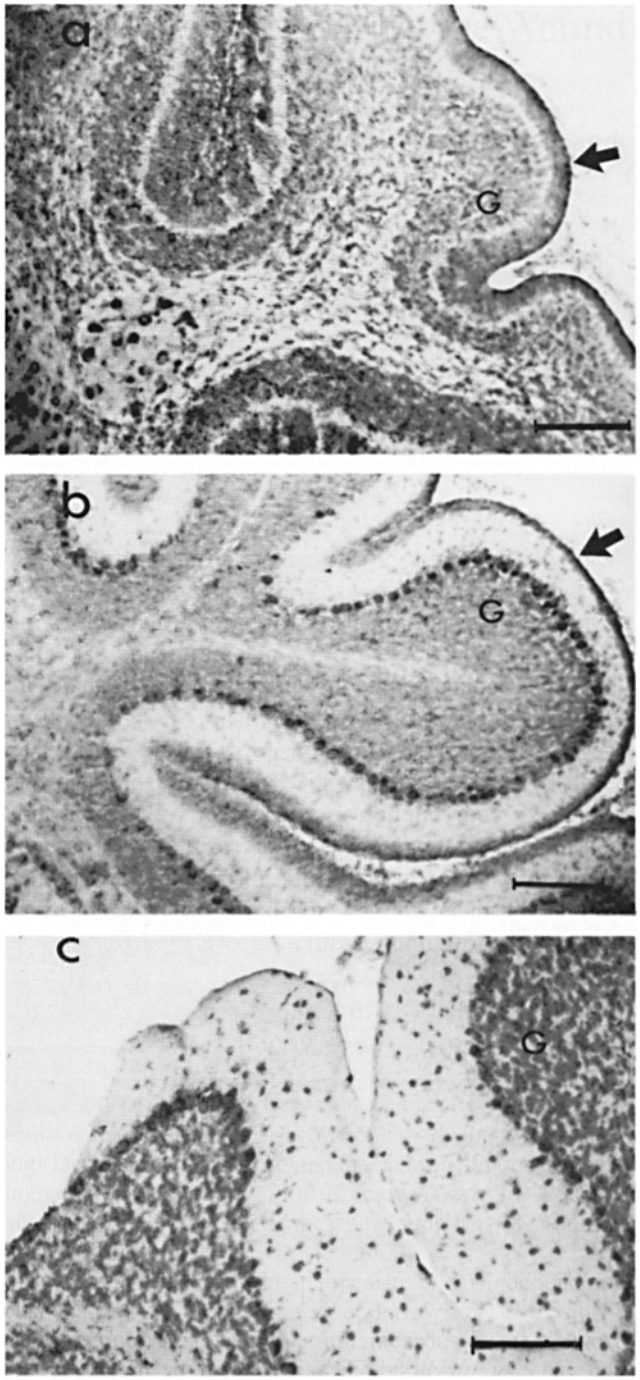

Figure 6. Developmental expression of MMIα in the mouse cerebellum. In situ localization of MMIα transcript in the cerebellum from P7 (*a*), P12 (*b*), and adult mice (*c*). The superficial layer of cells in the P7 and P12 EGL of the P7 and P12 mice (*arrows*) is more intensely labeled than the deeper layers of this structure. *G*, granular layer. Bar, 200 μm.

Discussion

Evidence for a Family of Mammalian Myosin I Genes

The characterization of MMIα, MMIβ, and MMIγ provides strong evidence for the existence of three novel mammalian myosin I genes. These are the first vertebrate myosin I genes described that are expressed outside the intestinal brush border. The classification of these predicted proteins as myosin I's is based on both a high degree of overall homology and the presence of highly conserved domains: some of these are shared by all myosins and others are unique to myosin I's. The latter point is exemplified by the conserved residues, QA.yaRDALAK.iYSR, first described as adjacent to the Acanthamoeba myosin I phosphorylation site (Pollard et al., 1991). Given the presence of multiple myosin I genes in at least two invertebrates, the existence of a family of mammalian myosin I genes is not unexpected.

We screened 42 subcloned PCR products to identify these three novel myosin I genes. While this is not an exhaustive search for myosin I family members, this result may suggest that these are the only products that can be obtained from these cell sources given the constraints of the PCR strategy we used. These constraints also would prevent the amplification of unconventional myosins like NinaC (Montell and Rubin, 1988) and the high molecular weight myosin I from Acanthamoeba (Horowitz and Hammer, 1990).

MMIβ may be the rat homologue of a newly identified myosin I purified from bovine adrenal glands (Barylko et al., 1992). 12 residues, LSVIDFTEDEVE, have at least 75% identity with a microsequenced fragment from the bovine myosin. A more recent paper from this group (Wagner et al., 1992) provides localization data for this protein in many tissues and cell types that overlap with expression of the MMIβ transcript.

Expression Pattern of MMIα, MMIβ, and MMIγ

MMIα is highly expressed in the brain, lung, and liver, while

MMIβ and MMIγ are detected in diverse tissues including the lung, kidney, small intestine, adrenal gland, and brain. These patterns of expression are distinct from one another and also substantially different from that of BBMI, which appears by both Northern blot and immunochemical analysis to be expressed only in the brush border of the small intestine (Hoshimaru et al., 1989; Bikle et al., 1991). The very different cellular distribution of these messages is consistent with the possibility that each of these products performs separate and unique functions.

The various messages detected by probes derived from MMIα, pGMMIβ, and pGMMIγ point to a further complexity within the mammalian myosin I family. One potential concern in interpreting these results is the crossreactivity that may occur by using myosin head sequence as the cDNA probe. However, hybridization was carried out at high stringency, and thus we expect that the bands correspond to alternate transcripts of the same gene or to ones that are highly homologous. This is supported by the different tissue distribution detected with probes to the three different genes. For MMIβ and MMIγ, this is further supported by the S1 nuclease protection results. For MMIα, the chance of crossreactivity is most unlikely because similar results were obtained using multiple probes, including those containing tail and 3′ untranslated sequences.

Three messages are detected with MMIα probes. Two of the messages appear to differ by alternate polyadenylation sites. The modifications responsible for generating the largest 6.5-kb message may include an additional coding sequence representing a separate functional domain, or this band may represent unprocessed nuclear RNA. An example of a myosin gene encoding multiple proteins is ninaC, in which the two transcripts differ by the presence of the COOH-terminal membrane binding domain (Porter et al., 1992). Using probes derived from pGMMIβ and pGMMIγ, we have detected a complex pattern of bands primarily in tissue; the cell lines examined typically displayed only a subset of these messages, suggesting that certain transcripts may be differentially expressed within multiple cell types in a single tissue.

MMIα Encodes a Protein Structurally Similar to BBMI

The cDNAs we isolated for MMIα encode a myosin I containing three functional domains: a canonical myosin head that contains sites for ATP hydrolysis and actin binding, a neck that possesses sites for calmodulin binding, and a positively charged tail that is likely to participate in membrane binding.

Calmodulin is the light chain for BBMI, for the unconventional myosin p190 (Espreafico et al., 1992), and probably for two other vertebrate myosin I proteins that have recently been purified (Coluccio, 1991; Barylko et al., 1992). Association of calmodulin with the heavy chain has been shown to regulate the activity of BBMI (Coluccio and Bretscher, 1987; Collins et al., 1990; Mooseker et al., 1989; Swanljung-Collins and Collins, 1991). The unconventional myosins p190 (Espreafico et al., 1992), dilute (Mercer et al., 1991), and yeast myo2 (Johnston et al., 1991), as well as BBMI, all encode a region in the neck of the protein that contains four to six related sequences of ~23 amino acids. These elements are homologous to the well-characterized calmodulin binding domain of neuromodulin (Chapman et al., 1991*a*,*b*). Like neuromodulin, BBMI and p190 bind calmodulin in the absence of Ca^{2+}. Within the analogous neck region, the predicted MMIα protein contains five calmodulin binding sites, and thus also probably binds calmodulin in a Ca^{2+}-independent manner. While these five sites possess the common motifs that appear necessary for calmodulin binding, each is more closely related to the homologous site in BBMI than to any of the other four sites within MMIα (and vice versa). This suggests that each site has been uniquely conserved. Moreover, these differences between sites may be reflected by biochemical data for BBMI that demonstrate a complex interaction between Ca^{2+} concentration, the number of calmodulins bound, and motor activity.

The neck region of MMIα also encompasses a 29 amino acid stretch that has high homology, based on sequence and location within the molecule, to a 29 amino acid calmodulin binding insert encoded by a rare transcript of chick BBMI (Halsall and Hammer, 1990). Interestingly, this stretch is encoded by all three MMIα cDNAs we have isolated and sequenced. However, it is possible that there may be additional splice products that vary in this region.

The COOH-terminal region of the predicted MMIα protein contains a high net positive charge. In both BBMI and Acanthamoeba myosin I's this region is also highly basic and has been implicated in the binding of these proteins to the plasma membrane. This interaction is mediated at least in part by a high affinity for negatively charged phospholipids found on the inner leaflet of the membrane (Adams and Pollard, 1989; Zot et al., 1992). Thus, MMIα protein would be expected to link the actin cytoskeleton to the cell membrane. Comparison of MMIα to both bovine and chicken BBMI reveals conserved domains within the tail (Fig. 1 *B*). Interestingly, 65% of these residues are not charged. In contrast, overall charge, but not specifically these domains, is conserved between MMIα and the equivalent regions in Acanthamoeba or Dictyostelium myosin I's.

We have considered here as myosin I all myosins that possess the positively charged membrane binding domain and do not contain any heptad repeats for dimerization, including all the Acanthamoeba and Dictyostelium myosin I's and BBMIs. This excludes such molecules as dilute, ninaC, and myo2, which have been classified within a broader category as "unconventional" myosins (Cheney and Mooseker, 1992). Even this narrow classification for myosin I may permit potential subdivisions. The 65% overall homology of MMIα to BBMI, as compared with 71% between the chick and bovine BBMIs, suggests a high degree of relatedness. This contrasts with the lower degree of homology (47–49%) that pGMMIβ and pGMMIγ show to the head domain of BBMI and to each other. Thus, MMIα and the BBMIs can be viewed as a subfamily within the myosin I family as outlined (Espreafico et al., 1992), while MMIγ and MMIγ would presently each be single members of separate groupings. Whether these similarities and differences extend to function remains to be determined.

Expression of MMIα in the Nervous System

In situ hybridization and Northern blot analysis reveals that MMIα mRNA is expressed in the mouse brain, from the neonate to the adult. Whereas virtually all brain neurons ex-

press MMIα mRNA, no message is observed in glial cells or in glial cell lines. We cannot, however, rule out the presence of MMIα mRNA in microglia or other relatively sparse populations of nonneuronal cells within the brain.

Although the MMIα message is present at all postnatal stages, expression varies both developmentally and regionally. For example, in situ hybridization in cerebellar granular neurons indicates that MMIα mRNA declines substantially from a peak between P7 and P12 to lower levels in the adult, whereas staining in hippocampal neurons remains equally strong throughout development. Northern blots reveal a general postnatal developmental decline in the expression of MMIα mRNA in the brain. Because most gliogenesis (in contrast to neurogenesis) occurs in the neonate, we suggest that the Northern blot data reflect summation of the actual decrease in average message levels per neuron and the dilution concomitant with the increasing relative percentage of glial RNA.

In addition to postmitotic neurons, neuroblasts in the external granular layer of the cerebellar cortex also stain for MMIα mRNA. These cells continue to divide postnatally; those fated to become granular neurons (the great majority) migrate past the Purkinje cells to form the internal granular layer (Altman, 1972). Interestingly, MMIα mRNA expression is highest in the outermost layer of the EGL (in contrast to the inner layers), which contains those cells that are still dividing and just beginning to extend bipolar processes. We do not yet know whether MMIα is expressed in other neuroblasts and how early this expression commences. It will be of considerable interest to explore these questions through examination of the embryonic brain.

We began our search for novel myosin I genes with the hypothesis that one or more of these motor proteins may participate in processes critical to neuronal development and function such as cell migration, neurite outgrowth, and vesicular transport. The presence of MMIα in all neurons examined and their absence from glial cell are consistent with these roles. The high sequence homology of MMIα with BBMI, as well as the high level of MMIα mRNA in neuroblasts just beginning to extend processes, may suggest a role for MMIα at the leading edge of these processes creating motive force between the plasma membrane and the actin cytoskeleton. Whether MMIα can function as a motor for growth cone-like motility is the subject of ongoing investigation.

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